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Protein fingerprints of single MCF7 breast cancer cells were mapped using both one-dimensional and two-dimensional capillary electrophoresis (CE). While one-dimensional CE can resolve dozens of proteins, two-dimensional CE has the potential to resolve hundreds or thousands of proteins with broad dynamic range. Cellular proteins are labeled on column by coupling lysine residues with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde. Labeled proteins are detected by laser-induced fluorescence in a sheath flow cuvette. Capillary sieving electrophoresis and capillary micellar electrophoresis were used to characterize proteins in single cells in one-dimensional separations, while the two techniques were combined to generate two-dimensional protein fingerprints from single cells.

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Introduction

Conventional protein analysis technology requires the protein content extracted from roughly 100,000 cancer cells. We are interested in developing technology that is five orders of magnitude more sensitive, so that we can characterize the protein content of a single cell. This project is driven by the goal of determining the cell-to-cell variability in protein expression, and using that information as a prognostic indicator, much as chromosomal ploidy is now used.

Most of the effort in this project was spent in developing the ultrasensitive protein analysis technology. Cellular proteins are labeled on column by coupling lysine residues with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde. Labeled proteins are detected by laser-induced fluorescence in a sheath flow cuvette. Capillary sieving electrophoresis and capillary micellar electrophoresis were used to characterize proteins in single cells in one-dimensional separations, while the two techniques were combined to generate two-dimensional protein fingerprints from single cells. That instrumentation was used to map protein fingerprints of single MCF7 breast cancer cells using both one-dimensional and two-dimensional capillary electrophoresis (CE).

Body

In our DoD grant proposal "The Single Cell Proteome Project – Cell-Cycle Dependent Protein Expression in a Breast Cancer Cell Line", we outlined a plan for the characterization of protein expression in single cells obtained from the MCF-7 breast cancer cell line. We proposed the use of one- and two-dimensional capillary electrophoresis (CE) and ultrasensitive laser-induced fluorescence detection to produce these expressed protein fingerprints. Our instrumentation is able to detect proteins at the zeptomole (1 zmol = 1 × 10^{-21}mol) level with a dynamic range of three orders of magnitude, so that many proteins present at the single cell level are detectable.

In the first phase of this project cells from the MCF-7 breast cancer cell line were cultured and cellular extracts were prepared. This phase of the project entailed optimization of cell lysis conditions. An optimized lysis buffer containing anionic and zwitterionic detergents was obtained.

We were then able to optimize the size-based separation of MCF-7 cellular homogenate proteins using capillary SDS-DALT electrophoresis (CSE). In this separation mode, proteins migrate through a polymer matrix with mobilities proportionate to their size. Several parameters including polymer type and concentration, buffer characteristics, and electric field strengths were optimized to maximize the number of resolved components in MCF-7 protein separations. Figure 1 shows the optimized size-based separation of an MCF-7 cellular homogenate. In this experiment we were able to resolve 45 proteins from the protein extract mixture.

We then optimized the hydrophobicity-based separation of cellular homogenate proteins using micellar electrokinetic capillary chromatography (MECC). Several factors, including buffer characteristics, electric field strength, and capillary dimensions were optimized in this separation mode. Figure 2 shows the optimized hydrophobicity-based separation of MCF-7 cellular homogenate proteins. In this experiment we were able to resolve 46 proteins from an MCF-7 protein extract.

Once the one-dimensional CE separations were optimized we were able to address the separation of MCF-7 homogenate proteins using two-dimensional CE. While one-dimensional CE can resolve dozens of proteins, two-dimensional CE has the potential to resolve hundreds or thousands of proteins when two orthogonal separation mechanisms are used. Figure 3 shows the instrument that was developed for these experiments. The instrumentation was described in detail by Michels et. al. [1, 2]. Early two-dimensional CE separations using MCF-7 cellular homogenates were performed using CSE in the first dimension and MECC in the second dimension. These fully-automated separations took at least four hours to complete. Improvements to the method were made to decrease the run time and to increase the number of resolved components. In recent experiments we have used capillary zone electrophoresis (CZE) in place of MECC in the second dimension, which has improved the resolution in our two-dimensional CE separations. In addition, by increasing electric field strengths and by decreasing both capillaries' lengths and inner diameters, experiments can now be completed in less than 90 minutes. Figure 4 shows an optimized two-dimensional CE separation of an MCF-7 cellular homogenate.

While breast cancer tumors consist primarily of epithelial-derived cancer cells, they also contain normal epithelial, endothelial, and fibroblast cells. Homogenate analyses thus reflect all cells present, not just the cells of interest. Additionally, many of the most harmful cells such as metastatic and chemotherapy-resistant cells are likely to be present in small proportions, and thus their expression may be obscured by more abundant proteins when analyzing a homogenate prepared from a biopsy. For these reasons we proposed the development of a method for the analysis of single breast cancer cells. Once we had developed the methodology for the separation of MCF-7 cellular homogenate proteins we then moved on to use these separation techniques with single MCF-7 cells. Some optimization of on-capillary lysis and labeling conditions was required in these experiments. Figure 5 shows the one-dimensional CSE separation of three single MCF-7 breast cancer cells as well as a blank (from the solution surrounding cells in an MCF-7 cell suspension). In doing these single-cell experiments we observed that the overall profiles were similar for each of the cells, however, some differences in overall and relative peak amplitudes were also evident. These differences are thought to be the result of cell-cycle dependent protein expression. However, this hypothesis has not yet been verified.

Figure 6 presents two-dimensional capillary electrophoresis data generated from single MCF-7 cells [3]. The top panel was generated from a cell grown under normal conditions and the second treated with 2.5 mM sodium butyrate for 48 h to induce apoptosis. Butyrate is an inhibitor of histone deacetylase, alters gene expression, inhibits DNA synthesis, and induces apoptosis. These landscapes share some features; a mesa is present at low molecular weight, that saturates our fluorescent detector and a ridge extends from that mesa to higher molecular weight. The cell treated to induce apoptosis presents a number of features that are either up- or down-regulated compared to the untreated cell. Single-cell protein fingerprinting allows study of the cell-to-cell variation in response to treatment, which can provide insight into those factors that determine the response of a particular cell to treatment.

We had also planned to characterize the MCF-7 cell-cycle dependent protein expression. That goal has proven to be overly ambitious. The development of technology to monitor protein expression from single cells is unprecedented, and has required much effort and time. Now that we are able to characterize protein expression in single cells, we anticipate that we will generate cell-cycle dependent protein fingerprints from single cells within the next six months.
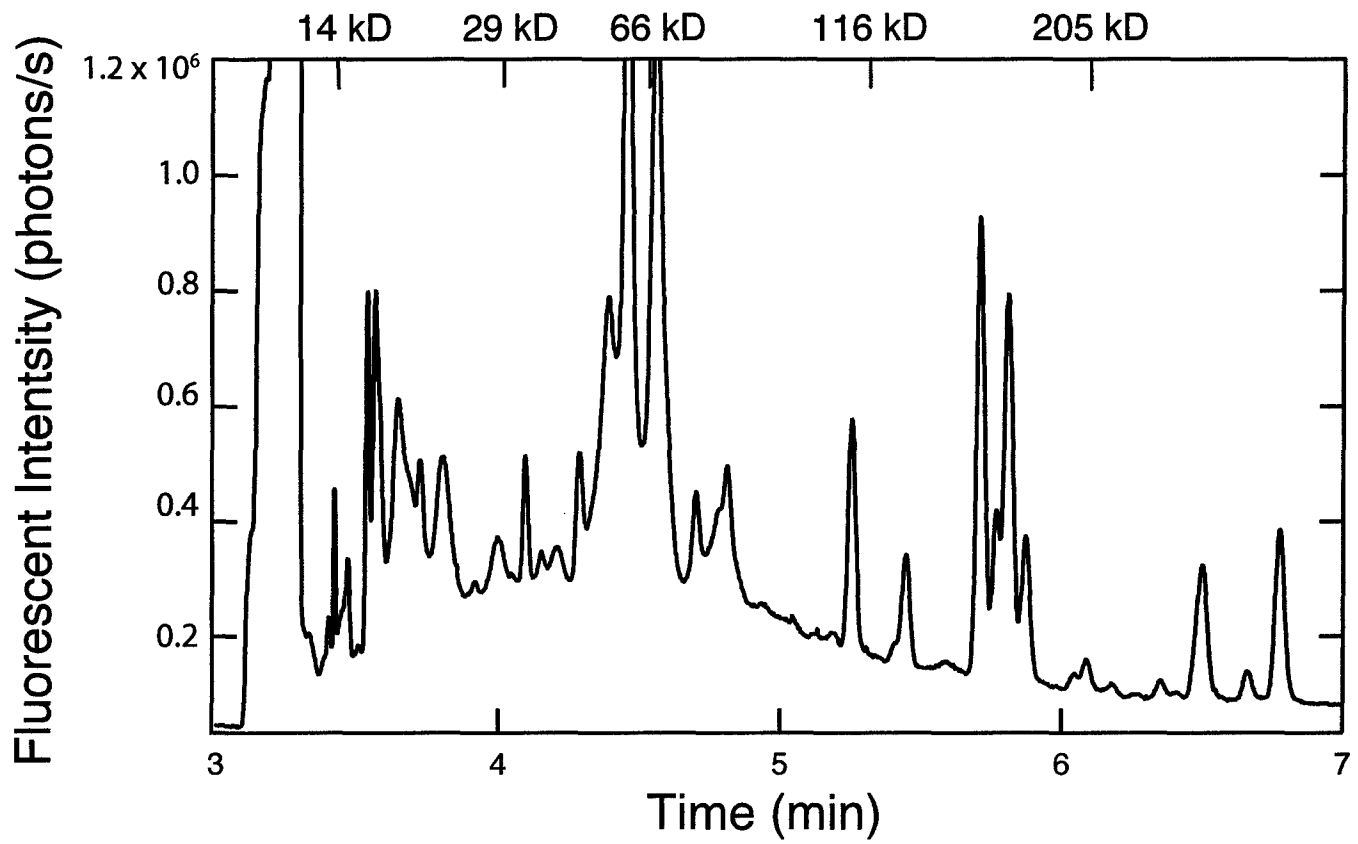
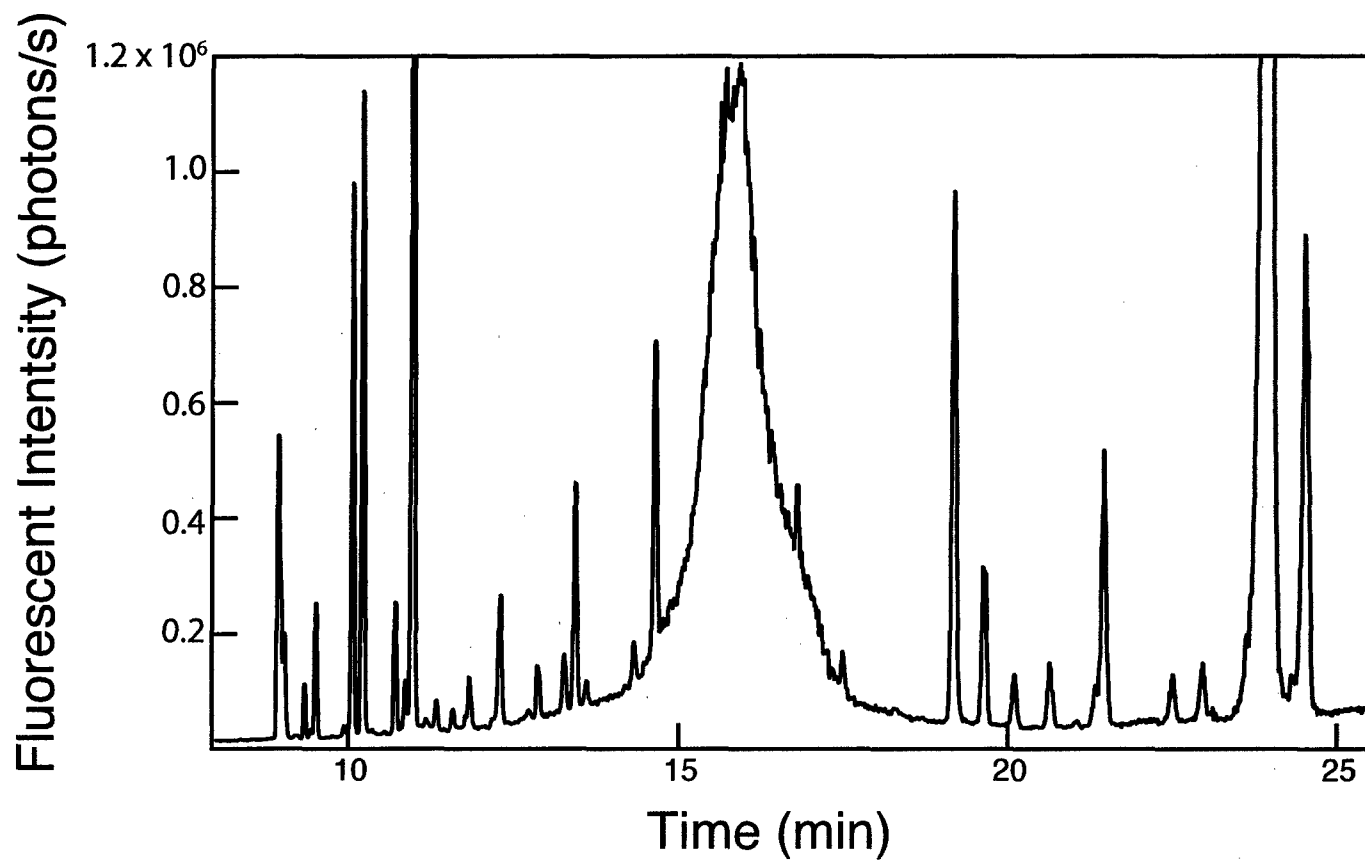Figure 1. Capillary SDS-DALT electrophoresis separation of MCF-7 cellular homogenate.

Figure 2. Micellar electrokinetic capillary chromatography separation of MCF-7 cellular homogenate.
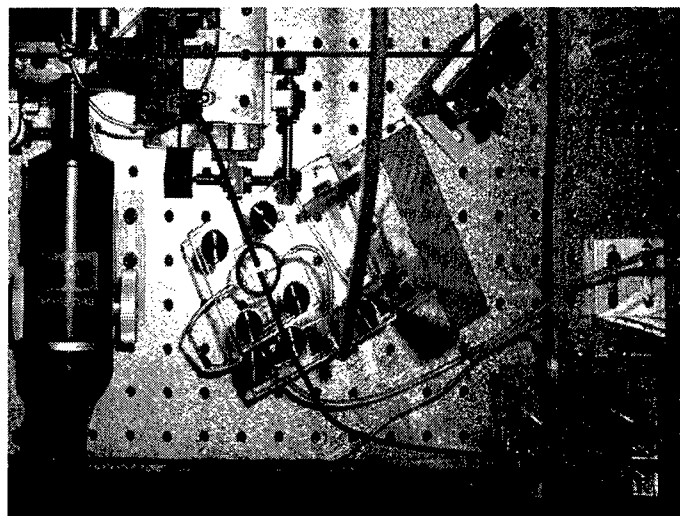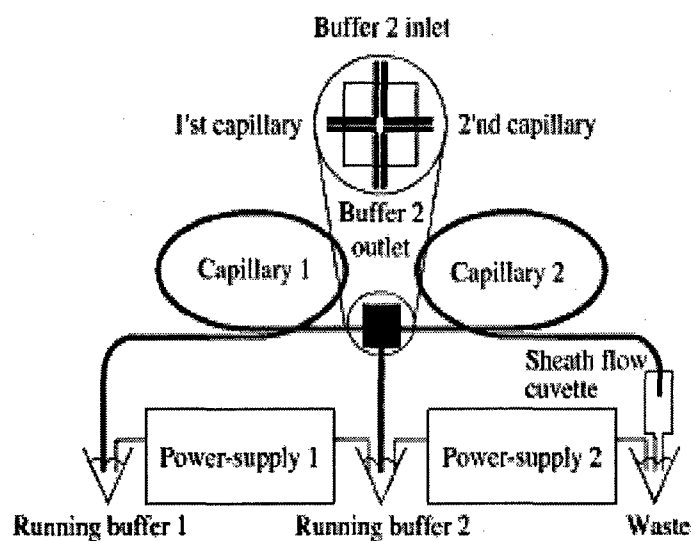
Figure 3. Two-dimensional capillary electrophoresis setup. 3a shows the instrument schematic. 3b shows a photo of the setup including the injection block (bottom right), the capillaries (shown in red), the laser line (shown in green), the interface between the two capillaries (center), and the sheath flow cuvette (upper left).
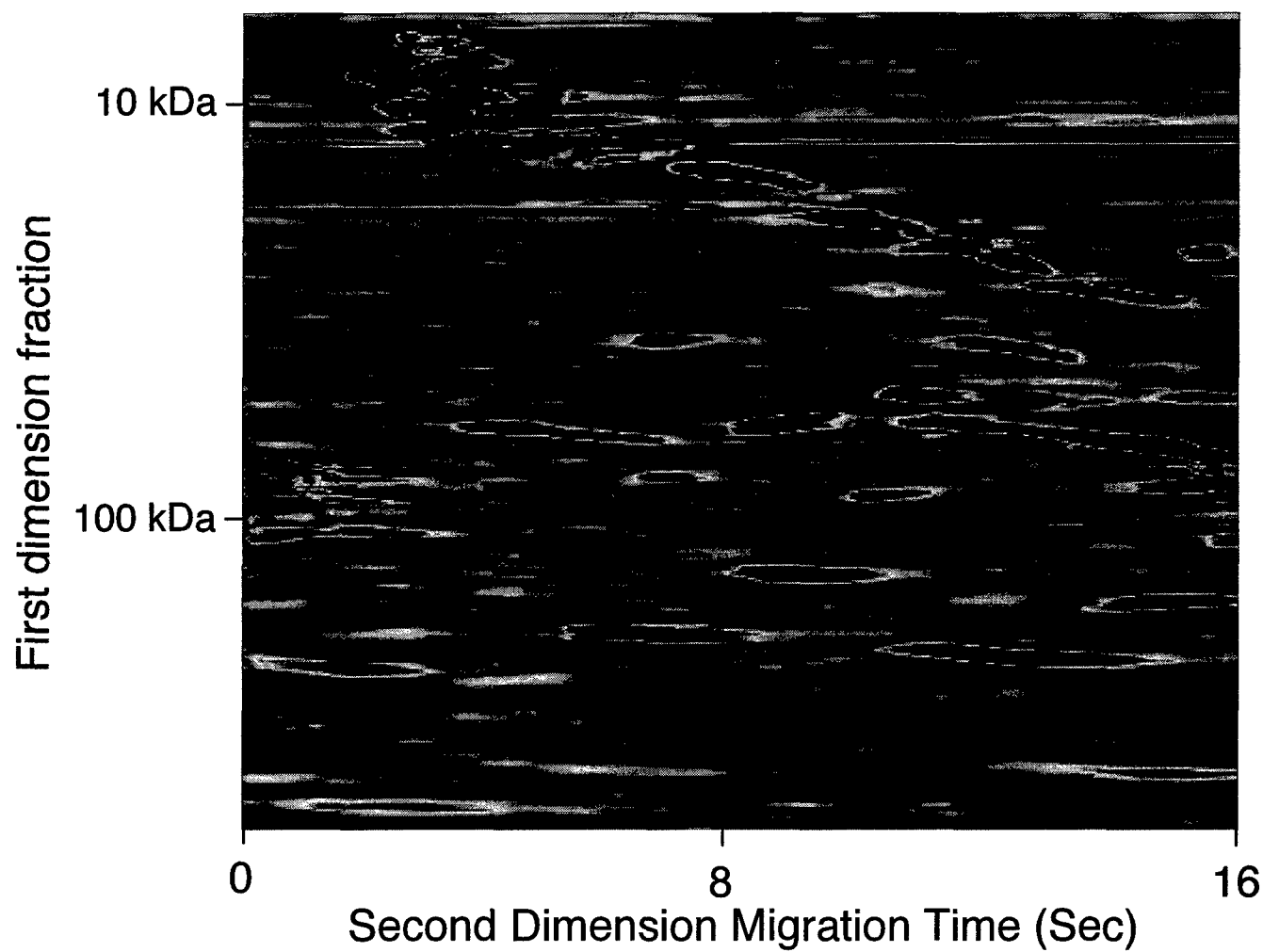
Figure 4."Gel view" of two-dimensional capillary sieving electrophoresis-capillary zone electrophoresis separation of MCF-7 cellular homogenate.

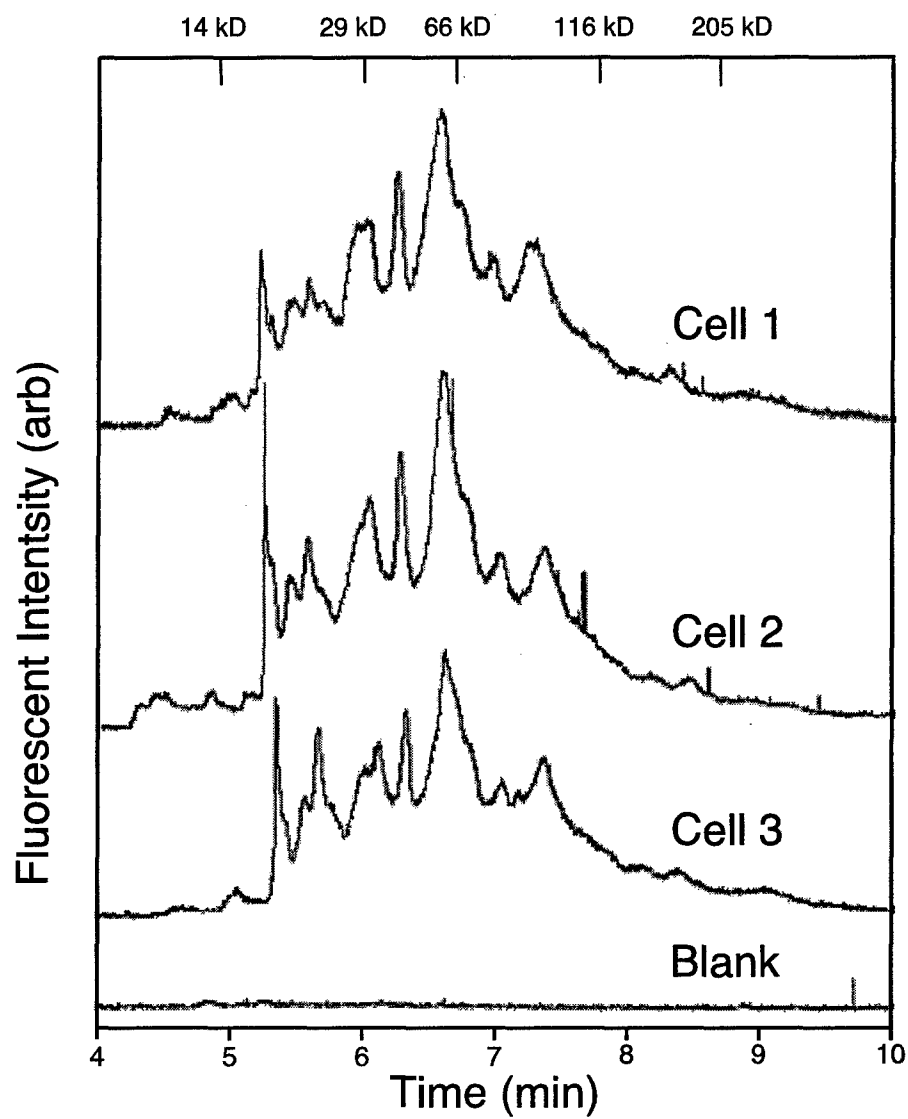Figure 5. Capillary SDS-DALT electrophoresis separation of three single MCF-7 cells, and a blank experiment (from the solution surrounding cells in a cell suspension).
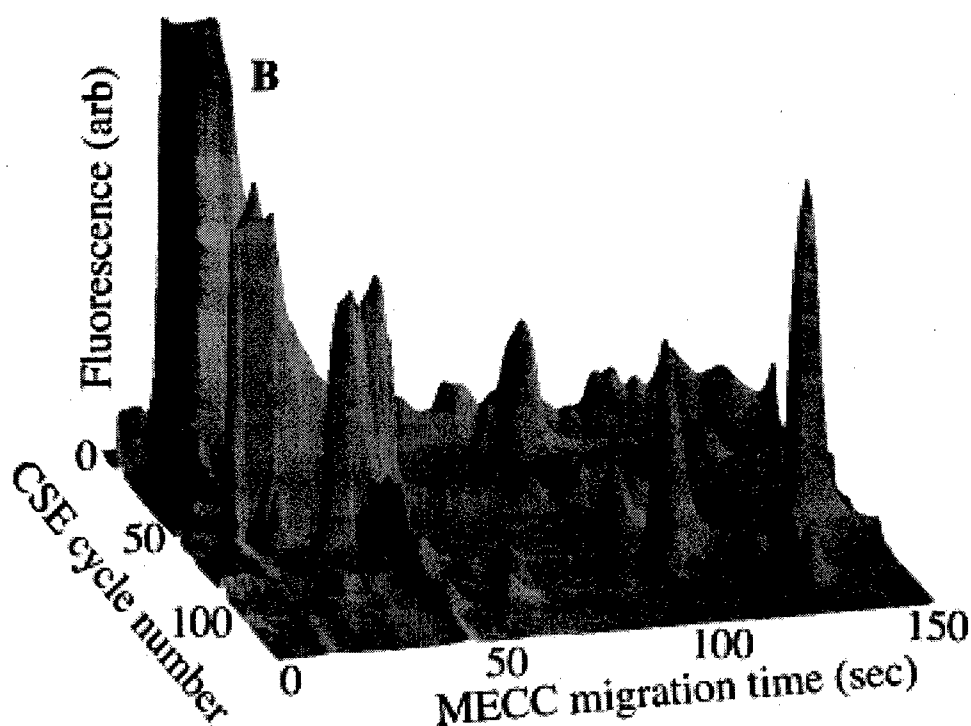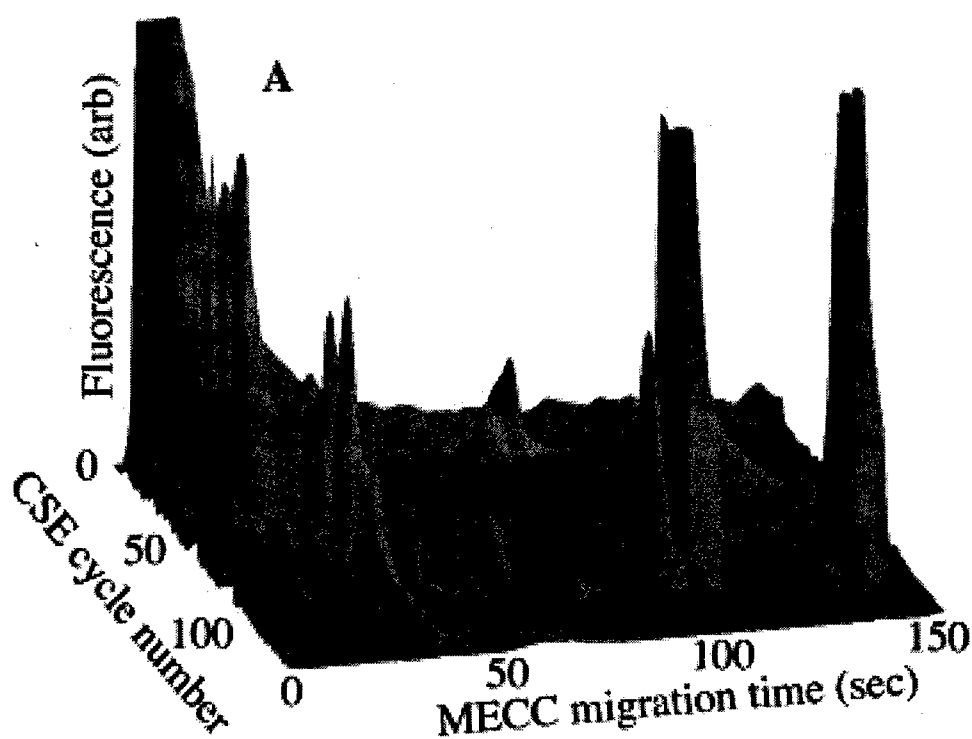
Figure 6. Two-dimensional capillary electropherograms generated from single MCF-7 cells. The top panel (A) was obtained from a cell cultured under normal conditions. The bottom panel (B) was obtained from a cell cultured with butyrate for 48 hours.

Key Research Accomplishments

•   Developed protein electrophoresis instrumentation that is five orders of magnitude more sensitive than conventional technology.

•   Generated first one-dimensional protein electropherograms from single breast cancer cells

•   Generated first two-dimensional protein electropherograms from single breast cancer cells

Reportable outcomes

Publications:

1. "Capillary sieving electrophoresis/micellar electrokinetic capillary chromatography for two-dimensional protein fingerprinting of single mammalian cells" S. Hu, D. Michels, M. A. Fazal, C. Ratisoontorn, M.L. Cunningham, N.J. Dovichi, *Analytical Chemistry 76*, 4044-4049 (2004).

2. "Chemical Cytometry" N.J. Dovichi, S. Hu, *Current Opinion in Chemical Biology 7*, 603–608 (2003).

Presentations:

2003

   14th International Symposium on Pharmaceutical and Biomedical Analysis, Orlando September.

   Princeton University, Chemistry Department, Princeton October

   American Associated of Pharmaceutical Scientists, Annual Meeting, Salt Lake October

   NSF/NIH Workshop on Nanobiotechnology, Arlington October

   Eastern Analytical Conference, Somerset November

   Allen Institute for Brain Science, Seattle November

   23rd International Symposium on the Separation of Proteins, Peptides and Polynucleotides, Delray Beach, November.

2004

   Hyphenated techniques in chromatography (HTC-8), Brugge February

   Pittsburg Conference, Chicago March

   3rd Symposium Institute for Systems Biology, Seattle 2004 April.

   6th Central New England Chromatography Council/Greater Boston Mass Spectrometry Discussion Group Symposium, Boston, May.

   1st Annual Symposium on Enabling Technologies for Proteomics, Montreal, May.

   27th International Symposium on Capillary Chromatography, Riva del Garda Italy June

   Lund University, Department of Technical Analytical Chemistry, Lund Sweden June

   Interact 2004, Gold Coast Australia, July.

   Münster Conference on Single Cell Analysis, Münster, November.

   University of Virginia, Charlottesville October

   Bucknell University, 2004 November.

Conclusions

We have obtained the first one- and two-dimensional fingerprints of protein expression in single breast cancer cells. This technology provides an unprecedented glimpse into the make-up of individual cells, which may have value in generating additional information on which to base prognosis.

Our long-term goal is to obtain single-cell protein fingerprints from hundreds of cells obtained from a breast cancer biopsy, and to use those fingerprints as prognostic markers to guide therapy. This project represents the first steps down a long road; we have developed the ultrasensitive protein electrophoresis instrumentation that is required to map protein expression in single cells.

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Capillary Sieving Electrophoresis/Micellar Electrokinetic Capillary Chromatography for Two-Dimensional Protein Fingerprinting of Single Mammalian Cells

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We have developed a two-dimensional capillary electrophoresis method for the study of protein expression in single mammalian cells. The first-dimension capillary contains an SDS–pullulan buffer system to perform capillary sieving electrophoresis, which separates proteins based on molecular weight. The second-dimension capillary contains an SDS buffer for micellar electrokinetic capillary chromatography. After a 6-min-long preliminary separation, fractions from the first capillary are successively transferred to a second capillary, where they undergo further separation by MECC. Over 100 transfers and second-dimension separations are performed over an ~3.5-h-long period. We demonstrate this technology by generating protein fingerprints from single native MC3T3-E1 osteoprogenitor cells and MC3T3-E1 cells transfected with the human transcription regulator TWIST. We also present single-cell protein fingerprints from MCF-7 breast cancer cells before and following treatment to induce apoptosis.

Cytometry refers to the characterization and analysis of cells and cellular components. Modern technology is based on flow cytometry, wherein cells are treated with fluorescent stains and characterized by the fluorescence and light-scatter signals generated as the cells pass single file in a flowing stream through a focused laser beam.[1] Chemical cytometry employs microscale separation methods to analyze the composition of single cells. Early examples employed microscale electrophoretic separation with microscopic detection,[2–5] whereas more recent experiments employ capillary chromatography and electrophoresis

coupled with laser-induced fluorescence, electrochemical, or mass spectrometric detection for the study of amino acids, enzymes, neurotransmitters, oligosaccharides, and transmitters from single cells and organelles.[6–29] In parallel, there has been a thriving community focusing on the characterization of mRNA levels in single cells; these measurements are performed with reverse-transcriptase PCR and DNA sequencing or hybridization array technologies.[30,31]

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Appendix 1

We have employed one-dimensional (1-D) capillary electrophoresis for the characterization of proteins in single mammalian cells and in a *Caenorhabditis elegans* zygote.[23-29] In those experiments, proteins were labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), which reacts with the ϵ-amine of lysine residues, creating a highly fluorescent product. In the presence of an ionic surfactant such as sodium dodecyl sulfate (SDS) or sodium pentasulfate, FQ-labeled proteins produce high-efficiency peaks during capillary electrophoresis.

While providing rapid analysis of the protein content of single cells, 1-D separations are inherently limited in their resolution of complex mixtures. That resolution can be increased by combining two or more separation methods. As Giddings pointed out, the spot capacity of a multidimensional separation is given by the product of the peak capacity for the 1-D separations, assuming that the separations are not correlated.[32] O'Farrell's two-dimensional (2-D) electrophoresis system based on isoelectric focusing and SDS—polyacrylamide gel electrophoresis (SDS—PAGE) is the classic example of a multidimensional separation method for protein characterization.[33] However, micrograms of protein obtained from millions of cells are typically required to generate the sample for analysis.

Neukirchen reported a miniaturized 2-D electrophoresis system that could resolve ~100 components from a single *Drosophila* egg.[5] This system was based on O'Farrell's classic 2-D system performed on an ultrathin gel. Manipulation of the cells was crude: "Single eggs were squashed between Parafilm sheets and sucked into a capillary containing (an isoelectric focusing buffer)". Detection was by silver staining, with detection limits of ~2 pg of protein/spot, which corresponds to 0.1 fmol of most proteins. A *Drosophila* egg is typically 100 μm in radius and has a mass of 4 μg, which is 10 000 times larger than a typical mammalian cell.[34]

We have developed an automated 2-D capillary electrophoresis system for the characterization of complex protein samples.[35,36] This system is reminiscent of Jorgenson's sequential liquid chromatography/capillary electrophoresis separation of peptides.[37-40] In our system, FQ-labeled proteins undergo preliminary separation in a first-dimension capillary. Fractions are then successively transferred to a second capillary, where the proteins undergo a second-dimension separation. In our first experiment, the two separations were based on submicellar capillary electrophoresis at pH 7.5 and 11.1, respectively, in the two dimensions.[35] More recently, we have combined capillary sieving electrophoresis (CSE) and micellar electrokinetic capillary chromatography (MECC) for two-dimensional protein separation of cellular homo-genates.[36] In this paper, we report the use of the CSE—MECC technique to characterize the protein expression in single mouse osteoprecursor cells and cells that have been transfected with a transcription factor. We also present the 2-D electrophoretic analysis of proteins in single human breast cancer cells under normal growth conditions and cells treated to induce apoptosis. These cells are ~10 μm in diameter and contain 4 orders of magnitude less protein than the only other 2-D separation of proteins from a single cell.

EXPERIMENTAL SECTION

2-D Capillary Electrophoresis Instrument. The 2-D instrument is described elsewhere.[36] Briefly, separations were preformed in two fused-silica capillaries with 50-μm inner diameter and 138-μm outer diameter. Both capillaries were coated with linear polyacrylamide to reduce electroosmosis.[25] For the analysis of MC3T3 cells, the first-dimension capillary (capillary 1) was 30 cm long, and the second-dimension capillary (capillary 2) was 40 cm long. For the analysis of MCF-7 cells, both capillaries were 30 cm in length.

Two running buffers were used for the 2-D capillary electrophoresis experiments. Running buffer 1 contained 0.1 M TRIS, 0.1 M CHES, 6% pullulan, and 0.1% SDS (pH 8.6); the pullulan acts as a sieving matrix in a CSE separation. Running buffer 2 contained 0.1 M TRIS, 0.1 M CHES, and 20 mM SDS (pH 8.6) and was used for MECC separation.

Laser-Induced Fluorescence. Fluorescence detection with a sheath-flow cuvette was used to monitor the labeled proteins.[41-43] A 12-mW, 488-nm argon ion laser beam provided excitation. Fluorescence was collected with a 60×, 0.7 NA microscope objective, filtered with an Omega 630DF30 band-pass filter, and then detected with a Hamamatsu 1477 photomultiplier tube, which was biased at 1000 V. The detector was held at ground potential for operator safety.

Standard Proteins. A mixture of seven proteins (myoglobin, lysozyme, carbonic anhydrase, ovalbumin, bovine serum albumin, apotransferrin, phosphorylase *b*) was prepared at 8 nM concentration of each protein. The mixture was injected at −200 V/cm for 5 s.

MC3T3 Cell Culture. MC3T3-E1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Immediately before single-cell analysis, cells were harvested by treatment with trypsin—EDTA for 5 min. Cells were thoroughly washed with phosphate-buffered saline (PBS) for three times to remove the medium before single-cell analysis.

TWIST Transfection. A cDNA clone of human TWIST was subcloned into pcDNA3.1/V5-His TOPO TA vector (Invitrogen Corp.). Stable transfection of MC3T3 cells was performed with SuperFect transfection reagent (Qiagen). The cells were selected and expanded in medium containing Geneticin (Gibco BRL).

MCF-7 Cell Culture. MCF-7 human breast cancer cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 UI/mL streptomycin, 100 μg/mL penicillin, and 50 μg/mL gentamycin. Immediately before

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Appendix 1

single-cell analysis, cells were thoroughly washed with PBS for three times to remove the medium.

MCF-7 cells were treated with 2.5 mM sodium butyrate for 48 h to induce apoptosis. The cells were harvested and washed with PBS for three times before analysis.

Single-Cell Protein Fingerprinting. Cells were washed three times with PBS to remove residual growth medium, which would otherwise generate a background signal that would swamp the single-cell protein electropherogram. Immediately before the single-cell analysis, KCN was added to the cell suspension to give a concentration of 2 mM. A drop of this cell suspension and ~50 μL of 10 mM FQ with 0.5% SDS (W/V) solution were placed at different locations on a microscope slide. The capillary tip was lowered into the drop of FQ and SDS, and an 11-kPa negative pressure was applied to the distal end of the capillary for 2 s to inject a plug of SDS and FQ into the capillary. The capillary tip was then centered over a cell. The cell was injected into the capillary by applying the negative pressure for 1 s. Finally, the capillary tip was moved to the drop of FQ and SDS. SDS–FQ was injected again for 2 s. The capillary tip was then placed in a vial containing 0.1 M TRIS, 0.1 M CHES, 6% pullulan, and 0.1% SDS (pH 8.6) that had been heated to 95 °C for 5 min to denature and label the proteins. After the labeling reaction was complete, the capillary tip was moved to a fresh vial of the same buffer.

2-D CSE×MECC Separation of Proteins from a Single Cell. After the labeling reaction was complete, a −300 V/cm potential was applied across capillary 1 for 6.0 min to perform a preliminary separation. Following the preliminary separation, a series of transfer and MECC separation cycles began. In each cycle, a fraction from capillary 1 was moved into capillary 2 by applying −300 V/cm across both capillaries for 6 s. Once the fraction was transferred, the second-dimension separation was initiated by applying −300 V/cm only across capillary 2. Voltage was applied to the inlet of capillary 1 to eliminate the voltage gradient and to prevent flow in capillary 1 during the second-dimension separation. The cycles of fraction transfer and second-dimension separation were repeated under computer control.

High voltages were driven using two 0–30 kV dc power supplies. Safety shields were used to protect the operator from high voltage.

Data Collection and Processing. Data were collected with a PC using software written in LabView. Data processing was performed on a Macintosh computer using software written in Matlab.[36]

RESULTS

CSE–MECC Separation of Standard Proteins. A set of seven standard proteins was analyzed with the 2-D capillary electrophoresis system. A set of seven spots was observed (data not shown). A plot of cycle number, which is proportional to the migration time of the protein in CSE, increased linearly with logarithm of molecular weight ($r = 0.996$). The sieving electrophoresis dimension of the two-dimensional separation provides an estimate of molecular weight similar to that provided by other sieving electrophoresis methods, such as SDS–PAGE.

2-D Protein Fingerprint from a Single Osteoprogenitor Cell. MC3T3-E1 osteoprogenitor cells were originally derived from fetal mouse calvaria and are at an intermediate stage between primitive stem cells and fully differentiated osteoblasts. These cells are typically 10 μm in diameter and provide a challenging target for chemical cytometry performed by 2-D capillary electrophoresis.

Figure 1 presents a single-cell 2-D electropherogram generated from one MC3T3 cell. Panel 1A presents the data in the form of an intensity plot, where the density at each point is proportional to the logarithm of the fluorescence signal. The logarithm helps compress the wide dynamic range of the data and also resembles the response function of photographic film, which is commonly used to record silver-stained gels. The image is overexposed to make visible some of the low-intensity spots. To provide information on the overexposed regions, a set of 10 filled contours is superimposed on the image.

Sections B and C of Figure 1 present the same data as a landscape image. In this case, the height at each point is proportional to the fluorescence signal. The data consist of a mesa at the 15th CSE cycle and 10 s in MECC migration time. This mesa undoubtedly consists of a number of low molecular weight proteins and peptides that comigrate. The signal from these components saturates our photomultiplier tube, which demonstrates the sensitivity of the instrument.

Ridges at 15, 83, and 110 s in MECC migration time are produced by system peaks. The latter two ridges are more visible in the overexposed intensity plot of Figure 1A. The sheath-flow cuvette produces a response to refractive index gradients, and these system peaks are likely caused by buffer components transferred from the CSE capillary.[44]

To highlight the dynamic range of the instrument, Figure 1C presents the same landscape, expanded by a factor of 10 in amplitude. A number of low-amplitude components in Figure 1B are observed as well-defined peaks in the expanded landscape. The signal-to-noise ratio of the 2-D separation exceeds 2000 and would have been larger if our detector had not saturated from the most intense component.

Spot Statistics. An unsupervised algorithm was used to identify the local maximums across the image, and a nonlinear regression analysis was used to fit a Gaussian surface to those maximums. Fit results that did not converge were rejected. A total of 167 local maximums were identified in the baseline-corrected data of Figure 1A.[36] The standard deviation of the Gaussian function was used to estimate the spot width in both the CSE and MECC dimensions. The mean spot width in the CSE dimension was 1.5 cycles and in the MECC dimension was ~2.3 s.

Peak capacity in one-dimensional separations is given by the duration of the separation window divided by 4 times the average peak standard deviation; in this case, our CSE separation produced a peak capacity of 27 and the MECC separation produced a peak capacity of 14.

The spot location in the CSE and MECC dimensions was uncorrelated ($r = -0.10$). For uncorrelated separations, the spot capacity is given by the product of the peak capacities in the one-dimensional separations. The spot capacity for this two-dimensional separation is ~375. Our observation of 167 different spots approaches the limit of this separation. Most spots undoubtedly contain many comigrating components.

Background. Cells are grown in a nutrient-rich medium that can contaminate the single-cell electropherograms. Also, the

(44) Cheng, Y. F.; Dovichi, N. J. *Can. J. Spectrom.* **1989**, *34*, 90–93.
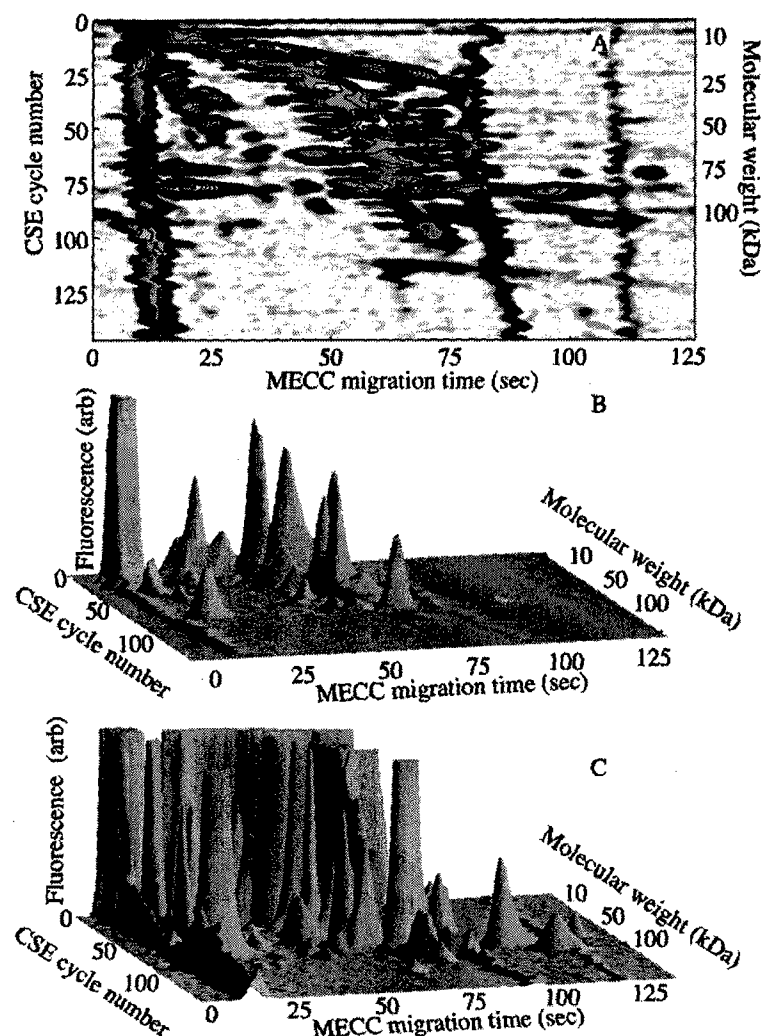
Figure 1. Protein landscape image from a single MC3T3-E1 cell. Panel A presents the data in an image plot where density is proportional to the logarithm of the fluorescent intensity. The image is overexposed to highlight some of the low-level proteins. A set of 10 equally spaced filled contours is superimposed on the overexposed region. Panel B presents the data in a landscape plot, where the height of the peak is proportional to the fluorescence intensity. Panel C presents a landscape plot where the data have been expanded by a factor of 10 to highlight some of the low-level peaks. Molecular weight is determined from the analysis of standard proteins.[36]
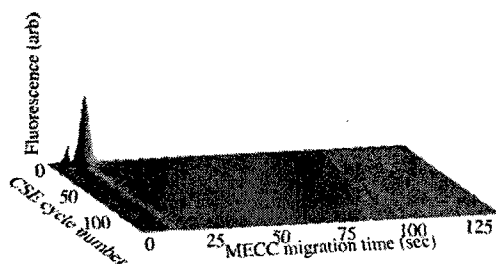

Figure 2. Background signal generated by aspirating the cellular supernatant.

cellular supernatant can be contaminated by proteins and other components produced from lysed cells. To minimize the contributions of the growth medium on the single-cell signal, we wash the cells with PBS solution before analysis.

Figure 2 presents the background signal generated by aspirating an aliquot of the cellular supernatant. This blank signal consists of a low-amplitude peak, which roughly corresponds to the mesa that saturates the detector in Figure 1, along with the

system peaks and a few very low amplitude components. Otherwise, the blank signal is quite featureless. The residual growth medium in the cellular supernatant makes a negligible contribution to the 2-D electropherogram.

Effect of Transcription Factor on Single-Cell Protein Expression. Figure 3 presents data generated from four MC3T3 cells. Sections A—C of Figure 3 were generated from single cells from the parent cell line; Figure 3A is the same data as Figure 1. The landscapes generated by these cells are quite similar. Modest differences in peak position will likely be due to day-to-day changes in the buffer composition or room temperature. Variations in protein expression are likely dominated by differences in the phase of the cell in the cell cycle.[22] A second source of variation in protein expression may arise because these cells tend to differentiate when in close contact, so that differences in distance to neighboring cells during culture should also generate differences in protein expression. A third source of variation may be due to stochastic variation in the number of regulatory proteins contained in the cell.[45]
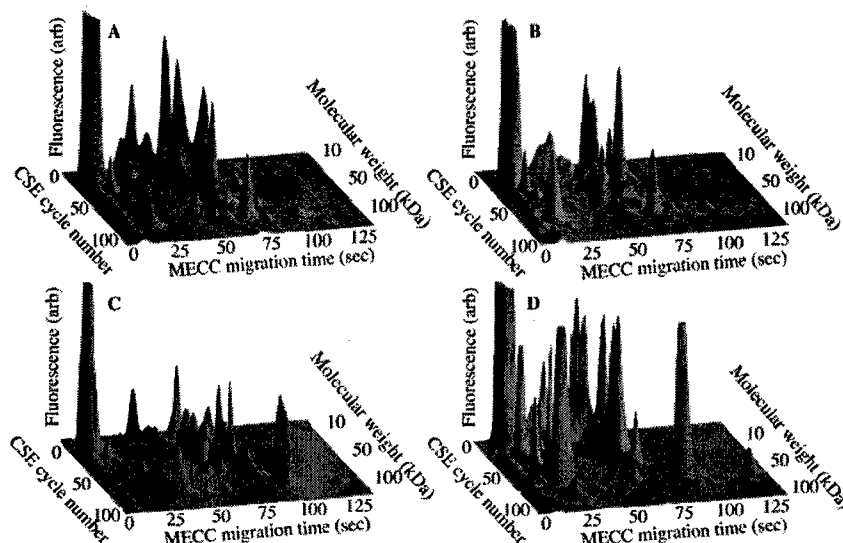
Figure 3. Protein landscape images from single MC3T3-E1 cells. Landscapes A—C were generated from untransfected cells. Landscape D was generated from a single MC3T3-E1 cell that had been transfected with the transcription regulator TWIST.

Figure 3D presents the landscape of a single cell from an MC3T3 cell line that has been transfected with the human transcription regulator TWIST. This 20-kDa protein is a basic helix—loop—helix protein that is involved in skeletal development, and its overexpression may inhibit osteoblast differentiation.[46,47] MC3T3 cells transfected with human TWIST demonstrate an altered cellular phenotype with a more cuboidal appearance. TWIST transfected cells generated much larger signals than the untransfected cells. A new 20-kDa component appeared in the protein fingerprint of the transfected cell; the molecular weight of this peak matches that of the human TWIST protein.

The overexpression of this single transcription regulator has a profound influence on both the visual appearance of the cell and on the single-cell electropherogram. This change in protein expression accompanying expression of TWIST is not surprising. The striking differences in the appearance of the cells with and without transfection can only arise from differences in the expression of highly expressed structural proteins; these structural proteins likely form a significant fraction of the proteins observed in the single-cell electropherogram.

The sensitivity of the single-cell protein fingerprint is notable. Seven components from the transfected cell generated peaks that saturated our fluorescence detector.

Single-Cell Protein Fingerprints from Cultured Breast Cancer Cells. As a second example, Figure 4 presents the protein fingerprint from a single MCF-7 breast cancer cell and from a cell that had been treated for 48 h with sodium butyrate. This inhibitor of histone deacetylase alters gene expression, inhibits DNA synthesis, and induces apoptosis.[48] These landscapes share some features with those generated by the osteoprogenitor cells;

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(46) Wolf, C.; Thisse, C.; Stoetzel, C.; Thisse, B.; Gerlinger, P.; Perrin-Schmitt, F. *Dev. Biol.* **1991**, *143*, 363—373.
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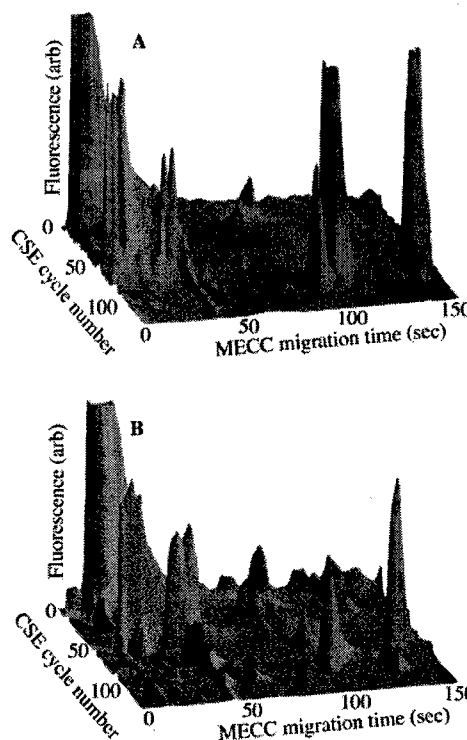(48) Chopin, V.; Toillon, R. A.; Jouy, N.; Le Bourhis, X. *Br. J. Pharmacol.* **2002**, *135*, 79—86.

Figure 4. Protein landscape images from single MCF-7 cells. Landscape A was generated from a cell grown under normal conditions. Landscape B was generated from a cell that had been treated with 2.5 mM sodium butyrate for 48 h to induce apoptosis.

a mesa is present at low molecular weight, and a ridge extends from that mesa to higher molecular weight. There are also many differences in the fingerprints, as expected from the different sources of the cells.

The cell treated to induce apoptosis presents a number of features that are either up- or downregulated compared to the untreated cell. Single-cell protein fingerprinting allows study of the cell-to-cell variation in response to treatment, which can

provide insight into those factors that determine the response of a particular cell to treatment.

CONCLUSIONS

We have demonstrated ultrasensitive protein analysis technology that provides high dynamic range for the multidimensional analysis of the minute amount of protein present in a single mammalian somatic cell. While we are pleased with the detection sensitivity of this technology, the resolution of the capillary two-dimensional falls far below that of classic two-dimensional gel electrophoresis, which is capable of resolving an order of magnitude more components. We have discussed causes of the loss of resolution in the single-cell experiment elsewhere.[36] These causes include diffusion of analyte in the CSE capillary during the long separation and the large transfer volume from the first to the second capillary, which degrades the performance in the MECC dimension. Work is underway to deal with these issues.

We should point out another potential improvement to the technology. We have reported three high-sensitivity LIF detectors for multiple capillary DNA sequencing.[49-51] Given adequate resources, those instruments could be modified for high-throughput single-cell protein fingerprinting. An automated 96-capillary instrument should generate thousands of 2-D single-cell capillary electropherograms per day of operation, which will facilitate systematic study of changes in protein expression in single cells associated with cancer progression, differentiation, and development.

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Appendix 2

ELSEVIER

Chemical cytometry
Norman J Dovichi* and Shen Hu

Chemical cytometry refers to the use of high-sensitivity analytical tools to characterize single cells. These tools include mass spectrometry, electrochemistry and capillary separation methods. This review focuses on the use of capillary electrophoresis coupled with high-sensitivity detection to characterize single cells. In survey experiments, biogenic amines and proteins have been characterized in single cells. In directed experiments, fluorescent substrates are used to monitor the activity of sets of enzymes, either within a family or along an enzymatic cascade. When combined with classical cytometry tools, it is now possible to monitor several cellular components in single cells as a function of cell cycle, which provides insight into the evolution of cellular composition as cells prepare for division.

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Abbreviations
CSE   capillary sieving electrophoresis
FQ   3-(2-furoyl)quinoline-2-carboxaldehyde
SDS   sodium dodecyl sulfate
SPS   sodium pentasulfate

Introduction

Cytometry refers to the automated measurement of physical or chemical characteristics of cells. The scope of the field is enormous; over 60 000 papers are indexed in Medline under the term. The field is usually divided into image and flow cytometry. Image cytometry involves automatic characterization of cellular information from a microscope image, whereas flow cytometry relies on spectroscopic and electrical characterization of single cells flowing in a fine stream.

Cytometry is important because cells differ in their composition and function, and understanding those differences is important in basic and clinical science. For example, cytometry is used to perform the fundamental step of classifying cells in complex tissues on the basis of specific cell-surface markers [1]. In the clinic, DNA ploidy is used as a prognostic indicator for some cancers,

where cells from differentiated tissue are diploid; replicating cells contain diploid, S-phase and tetraploid fractions; and some advanced tumors contain tetraploid and aneuploid cells [2].

Optical methods involving fluorescence staining are the workhorse technologies employed in cytometry. To be useful, these stains must be specific for their target. The great majority of cytometry measurements are performed with fluorescently labeled affinity probes, which do not demonstrate a change in spectral properties upon binding covalently to their target. The unbound reagents generate an unwanted background signal that is often reduced by washing the cells.

Fluorogenic stains are spectroscopically invisible until they react with or bind to their target. Classic examples are the DNA intercalating dyes, which are non-fluorescent until they bind to the hydrophobic interior of double-stranded DNA, upon which they become strongly fluorescent. A set of fluorogenic reagents is available to probe for specific enzyme activity; in this case, the reagents are non-fluorescent until a hydrolytic enzyme removes a functional group from the molecule, liberating a highly fluorescent product. More recently, molecular beacons have become popular [3]. These affinity reagents are synthesized from oligonucleotides tagged at one end with a fluorophore and at the other with a quencher. In the absence of target, the oligonucleotide forms a hairpin that holds the fluorophore and quencher in close proximity. The hairpin dissociates upon binding to the target, increasing the distance between fluorophore and quencher, which generates a fluorescent signal.

The number of properties that can be measured from a single cell depends on the number of spectral signatures that can be resolved and the number of reagents available. With conventional dyes, perhaps four or five different probes can be studied in the same cell. This limit is set by the rather broad emission of the dyes and the cost of the instrumentation to excite fluorescence at several wavelengths. There has been some interest in the use of quantum dots as spectroscopic labels; their narrow spectral features and common excitation wavelength might allow study of dozens of targets simultaneously [4] (see also the review by Frangioni, this issue).

Classic cytometry relies on use of a specific stain for each analyte and, as a result, suffers from one limitation: the unexpected is undetectable. By contrast, a suite of analytical methods has been developed for the specific goal of detecting and identifying components within a sample.

These tools include electrochemistry, mass spectrometry and capillary separation methods. It is now possible to monitor oligonucleotides, small molecules, proteins and enzyme activity in single cells. We refer to these techniques as chemical cytometry. This review focuses on the use of capillary separation methods in chemical cytometry.

Background

The use of separation methods to characterize the composition of a single-cell has a 50-year history [5–11]. The earliest study considered rRNA analysis in single cells using electrophoresis on a silk fiber [5]. The earliest single-cell protein analysis was a study of hemoglobin in single erythrocytes by electrophoresis through an acrylamide fiber, published in 1965 [6]. A series of papers was published in the 1960s and early 1970s on the analysis of single proteins in single cells [7–10]. Hyden reported the use of 200 µm diameter capillaries for the SDS–PAGE separation of nanograms of proteins from single cells [7]. Marchalonis reported the analysis of antibodies from single cells [8]. Wilson in 1971 and Ruchel in 1976 obtained electrophoretic separation of proteins from single giant neurons of *Aplaysia* [9,10]. Repin reported the characterization of lactate dehydrogenase isoenzymes in single mammalian oocytes by electrophoresis in 1975 [11].

Jorgenson inaugurated the modern era of single-cell analysis by using open tubular capillary chromatography for the amino acid analysis of a single giant neuron from a snail [12–14]. In one set of experiments, a cell was isolated, homogenized and centrifuged. The supernatant was injected directly onto the chromatography column and detected with amperometry [12]. However, only a small fraction of components are electroactive; a more universal detection scheme relied on maceration of the cell and fluorescent labeling of proteins with the fluorogenic reagent NDA. The reaction products were then injected into a capillary for analysis by either open tubular liquid chromatography or capillary electrophoresis [14].

Contemporaneously with Jorgenson, Ewing reported the use of an etched 10 µm inner diameter capillary to sample the internal contents of a single giant neuron. The same capillary was used for separation of biogenic amines by capillary electrophoresis with selective and sensitive detection by a carbon fiber microelectrode [15].

A few years later, Yeung reported the use of a specific reagent to derivatize glutathione, in individual erythrocytes [16]. The cell was treated with monobromobimane, which crossed the cellular membrane and reacted with cytoplasmic thiols; separation was by capillary electrophoresis and detection by laser-induced fluorescence. This experiment differed from Jorgenson's in that the reaction chemistry was performed on intact cells. Once the reaction was complete, a single cell was injected into the capillary and lysed.

Yeung's method required the use of a membrane-permeable reagent and Jorgenson's method required the manipulation of cellular products in a relatively large reaction chamber. In 1995, Ewing reported a more general approach, where the cell was injected into the capillary and lysed [17]. Fluorescent labeling chemistry was then employed within the capillary to derivatize biogenic amines in the cellular lysate. By performing the labeling chemistry within the 20 µm inner-diameter capillary, excessive dilution was avoided, and the entire reaction product was available for subsequent electrophoretic separation.

Single-cell protein electrophoresis
The sample size problem

There are several challenging issues when analyzing protein expression in single cells. Most importantly, protein samples are extremely complex. The human genome contains 30 000 genes. Alternative splicing and post-translation modifications generate perhaps 100 000 different proteins, which are present at extraordinarily low levels. Consider a typical somatic cell that is 10 µm in diameter. That cell has a volume of 500 fl and a mass of 500 pg. If the cell is 10% protein by weight, then there is only 50 pg of protein in a single cell. The average molecular weight of proteins in eukaryotes is about 30 kDa so that a single cell contains a total of about 2 femtomoles (10^9 molecules) of protein. We do not know the number of different types protein that are expressed in a single cell. If there are 10 000 different proteins expressed, then the average protein is present at the 200 zeptomole or 100 000 copy level (1 zeptomole = 10^{-21} mol = 600 copies) in a single somatic cell.

The multiple labeling problem

Detection of underivatized hemoglobin from single erythrocytes was first reported in 1965. Capillary electrophoresis with laser-induced fluorescence of this system was developed in the 1990s by Yeung's group [18,19]. Although able to detect highly expressed proteins, native fluorescence is of less value in the study of lower abundance proteins.

The minute amount of protein present in a single cell requires the use of extremely sensitive detection methods, and laser-induced fluorescence is the most sensitive detection technology available. Most commonly, derivatization chemistry is employed to label proteins with highly fluorescent tags.

Lysine is one of the most abundant amino acids, and the vast majority of proteins contain at least one lysine. Inspection of the yeast transcriptome reveals that 99.94% of open reading frames greater than 100 residues contain at least one lysine residue [20]. As a result, nearly all proteins can be fluorescently labeled with amine-reactive reagents.

The average yeast transcript codes for 20 lysine residues. Unfortunately, the labeling reaction does not, in general, go to completion, which results in the production of several reaction products. There are 2^N-1 fluorescent products generated by a molecule with N labeling sites [21]. For ovalbumin, which has 20 lysine residues and an acetylated N-terminus, we predict that there are 1 048 576 possible labeling products. The mobility of each product will differ, which results in very complex electropherograms from the labeling of a single protein. This variation in mobility arises from the neutralization of the cationic primary amine during the labeling reaction. In principle, it is possible to use a cationic labeling reagent, so that the overall charge of the protein is preserved. Unfortunately, the only cationic reagents are fluorescent, rather than fluorogenic, reagents. Impurities present in fluorescent reagents generate intense background signals that often obscure signals of interest.

Alternatively, it is possible to use a reagent that produces a neutral reaction product in combination with an ion pair reagent, which neutralizes unreacted residues. We discovered that the use of 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) to label proteins and addition of anionic surfactants, such as sodium dodecyl sulfate (SDS) and sodium pentasulfate (SPS), to the separation buffer results in very efficient separations, even in the case of incomplete labeling [22], presumably through ion-pairing with the unreacted lysine residues.

Chemical cytometry

Protein electrophoresis of single cells

Our group has employed fluorogenic reagents to label proteins from single HT29 adenocarcinoma cells. We follow Ewing's general approach. Like Ewing, we aspirate a cell into the capillary, lyse the cell, add a fluorogenic reagent, perform capillary electrophoresis to separate protein components, and detect with laser-induced fluorescence. In our case, we employ FQ. This reagent was developed by Novotny and is available from Molecular Probes [23]. It reacts with primary amines in the presence of a nucleophile, such as cyanide, to produce a fluorescent product. We observe that FQ preferentially reacts with the ε-amine of lysine residues, rather than with other primary amines.

Submicellar electrophoresis of proteins

Our first experiments were performed with submicellar capillary electrophoresis for the analysis of proteins expressed in single HT-29 colon cancer cells [24•]. In this form of electrophoresis, proteins were separated in an SPS-phosphate buffer. SPS is a surfactant, present below its critical micelle concentration. The SPS interacted with the labeled proteins, generating quite sharp peaks with over 400 000 theoretical plates. The separation window was quite wide, and 30 components were resolved from a single cell. Most components had reproducible migration times. However, one component demonstrated a significant mobility shift from cell-to-cell. We hypothesized that the component may have undergone post-translational modification, such as phosphorylation, which changed its charge and mobility. Although the migration time of the components was reproducible, the signal amplitude was not; the average relative standard deviation in peak height was 40% for the 10 most highly expressed proteins.

We presented several improvements to the instrumentation required for single-cell analysis, and we considered optimal surface treatments required for the analysis [25,26]. We also employed the same general technology to monitor protein expression in a single *Caenorhabditis elegans* zygote [27]. Roughly 30 components were resolved from this single-cell embryo.

Capillary sieving electrophoresis

We have recently developed a capillary sieving electrophoresis (CSE) protocol [28], which is the capillary equivalent of SDS–PAGE, for chemical cytometry analysis of single HT-29 cells [29,30,31•]. As in the submicellar electrophoresis experiment, roughly 25 components were resolved from a single cell. The molecular weight of these components ranged from ~10 kDa to over 125 kDa, the limit of the standard proteins used to calibrate the molecular weight axis. One component of ~12 kDa generated an intense signal that saturated our detector for all cells analyzed. Most cells also generated at least one other component that also saturated the detector. The noise in the baseline was about 5000-times smaller than the most intense signals, which allows us to study low-level protein expression in the presence of much more highly expressed proteins. Also like the submicellar electrophoresis experiment, the CSE data had reasonably good alignment of components between cells but poor cell-to-cell precision in peak amplitude; the relative standard deviation for the components was ~40%.

Cell-cycle-dependent protein expression

We hypothesized that the major source of cell-to-cell variation in protein expression was due to differences in the phase of the cell in the cell cycle. To test this hypothesis, we used the intercalating dye Hoechst 33342, which generates a fluorescence signal that is proportional to the amount of DNA within the cell [31•]. Cells were classified into different phases of the cell cycle on the basis of their Hoechst fluorescence, which was measured with a fluorescence microscope. Five diploid cells in the G1 phase were analyzed, as were five tetraploid cells that we classified as G2/M. Finally, one hexaploid cell was identified and analyzed. The ability to select unusual cells for further analysis is a hallmark of chemical cytometry.

An analysis of variance revealed that the majority of the cell-to-cell variation in protein expression was indeed due

to the phase of the cell in the cell cycle. The residual variation in peak height was ~20% for both the set of diploid and the set of tetraploid cells.

Diploid cells generated a signal that was half the amplitude of the signal from tetraploid cells. When normalized, the diploid and tetraploid single-cell electropherograms differed significantly for only one 45 kDa component. To identify this component, we prepared a large-scale SDS–PAGE separation of a cellular homogenate. We isolated the 45 kDa band, which co-migrated with the fraction of interest in capillary electrophoresis. We tentatively identified this component as cytokeratin 18 with the collaboration of our colleagues Rick Newitt and Ruedi Aebersold at the Institute for Systems Biology in Seattle, using in-gel digestion, mass-spectrometric analysis, and database searching [32]. This protein is the product of one of the most highly expressed genes in this cell line and undergoes a large increase in phosphorylation in the G2/M phases of the cell cycle. The phosphorylation increase leads to a mobility shift, which was observed in our single-cell electropherograms.

Chemical cytometry samples the entire cellular protein content, not just the cytosolic fraction. The single-cell electropherograms were similar to that produced from a cellular homogenate but were quite different from those produced from the cytosolic, membrane/organelle, nuclear and cytoskeletal/nuclear matrix fractions prepared from a cellular homogenate [30].

Only 20–30 components are resolved from a single cell, which is a small fraction of the entire protein content of the cell. Multidimensional separations produce superior resolution. We have developed a two-dimensional version of capillary electrophoresis for protein analysis [33]. We are in the process of employing this technology for the study of proteins from single cells, which will result in resolution of hundreds of components from one cell.

Metabolic cytometry
Chemical cytometry analysis of oligosaccharide biosynthesis and biodegradation

Metabolic cytometry is a form of chemical cytometry that monitors a cascade of biosynthetic and biodegradation products generated in a single cell [34]. In metabolic cytometry, a fluorescently labeled substrate is introduced into the cell by either endocytosis or microinjection. The cellular machinery acts upon the substrate, creating product molecules. Different product molecules are resolved by capillary electrophoresis separation. As long as the fluorescent label remains intact, the metabolic products can be tracked based on their fluorescence signal.

We collaborated with Monica Palcic, then at the University of Alberta, to monitor oligosaccharide metabolism in single HT29 colon cancer cells [34]. We treated the cells with a tetramethylrhodamine-labeled disaccharide $\beta Gal(1\rightarrow4)\beta GlcNAc$ substrate (LacNAc-TMR). The rhodamine provides a spectral signature that allows monitoring of metabolic products at the 150 yoctomole (1 yoctomole $= 10^{-24}$ mol) range [35]. LacNAc-TMR was taken up by cells with reasonable efficiency; an average of ~50 000 substrate molecules were taken up per cell. Both chemical cytometry and confocal microscopy were used to characterize the substrate uptake; both techniques demonstrate a ~40% relative standard deviation in the uptake of this reagent.

Confocal microscopy is unable to distinguish between metabolic products. By contrast, our single-cell capillary electrophoresis experiment detected five fluorescent products in addition to the starting material. Two biosynthetic products were observed, Lewis X (Le^X), a trisaccharide, and Lewis Y (Le^Y), a tetrasaccharide. Two biodegradation products were also observed, one was the monosaccharide GlcNAc and the other was the aglycone, which was produced by hydrolysis of the substrate. Finally, one component was observed that did not co-migrate with any of our standards and remains unidentified.

We performed metabolic cytometry on these cells to monitor oligosaccharide metabolism during the cell cycle. All cells showed biodegradation products. To our surprise, we observed that G1 phase cells were not biosynthetically active; based on the sensitivity of our instrument, fewer than 100 copies of Le^X or Le^Y were present per cell. More surprisingly, the G2/M phase cells fell into three classes. Some cells showed no biosynthetic product, some showed Le^X, and some Le^Y. No cells were observed to produce both the trisaccharide and the tetrasaccharide product at the same time. Production of Le^X and Le^Y proceed down different metabolic pathways. It appears that a single cell will commit to one or the other path, but not both.

We have also used this technology to monitor carbohydrate biodegradation in single yeast cells [36]. The greatest challenge in that experiment was developing tools to lyse single yeast cells; formation of spheroplasts with subsequent lysis by SDS was required.

Chemical cytometry analysis of kinase activity

Allbritton and co-workers have developed a metabolic cytometry method to monitor the activity of a suite of kinases [37,38•]. They synthesized a set of fluorescein-labeled peptides with the recognition site for protein kinase C, protein kinase A, calcium-calmodulin activated kinase II, and cdc2 protein kinase. A mixture containing a few hundred zeptomoles of each labeled peptide was microinjected into single rat basophilic leukemia cells. Like Yeung's and our work, the cells were lysed and the contents analyzed by capillary electrophoresis and laser-induced fluorescence.

Because of the high activity of kinase and phosphatases within single cells, it was necessary to rapidly lyse cells. A pulse from a Nd-YAG laser was focused near the cell on the microscope slide. The resulting acoustic wave from the laser pulse caused rapid lysis of the cell. An electric field was simultaneously applied to the capillary, injecting the cell's content and terminating any reaction within milliseconds.

The authors reported a detection limit of better than 10^{-20} moles, which is at concentrations less than native substrates so that competitive inhibition by the reporters is negligible. The authors measured constitutive kinase activity and activity in response to physiological and pharmacological agents.

Conclusions

Chemical cytometry employs powerful modern analytical tools to characterize the composition of single cells. The minute amount of analyte present in a single cell makes these analyses challenging. Work in the 1950s and 1960s employed microscopes to monitor the separation of highly abundant components from single cells. More recently, the development of ultrasensitive electroanalytical and, in particular, laser-induced fluorescence detectors has resulted in dramatic improvements in chemical cytometry.

Chemical cytometry offers a subtle advantage over classic analytical methods. Classic methods require sample lysis, followed by subsequent manipulations to prepare the sample for analysis. During the sample workup, hydrolytic enzymes can mix with the cytoplasm, resulting in degradation of some components. By contrast, we demonstrated that single cell analysis avoids this degradation because lysis is rapidly followed by electrophoresis, which separates the hydrolytic enzymes from their substrates [39]. Allbritton has carried this process to the extreme by using a pulsed laser to lyse cells while applying electrophoretic potential; hydrolytic enzymes are separated from substrate within milliseconds of lysis, reducing unwanted degradation of analyte to a negligible level [40].

Finally, this review focuses on capillary-based separations of macromolecules. Other chemical cytometry methods have been developed based on electrochemistry and mass spectrometry. The electrochemical methods focus on study of small molecules generated during exocytosis [41–45], whereas the mass spectrometric methods tend to focus on detection of peptides and low-molecular-weight proteins from single cells and tissues [46–50].

These technologies provide stunning capabilities to the biologist. As long as funding agencies continue to support the development of these new and powerful technologies, we will see a flourishing of applications of chemical cytometry throughout the biological sciences.

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